A tomato LATERAL ORGAN BOUNDARIES transcription factor, *SlLOB1*, predominantly regulates cell wall and softening components of ripening

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Fruit softening is a key component of the irreversible ripening program, contributing to the palatability necessary for frugivore-mediated seed dispersal. The underlying textural changes are complex and result from cell wall remodeling and changes in both cell adhesion and turgor. While a number of transcription factors (TFs) that regulate ripening have been identified, these affect most canonical ripening-related physiological processes. Here, we show that a tomato fruit ripening–specific *LATERAL ORGAN BOUNDRIES* (*LOB*) TF, *SlLOB1*, up-regulates a suite of cell wall–associated genes during late maturation and ripening of locule and pericarp tissues. *SlLOB1* repression in transgenic fruit impedes softening, while overexpression throughout the plant under the direction of the 35s promoter confers precocious induction of cell wall gene expression and premature softening. Transcript and protein levels of the wall-loosening protein EXPANSIN1 (*EXP1*) are strongly suppressed in *SlLOB1* RNA interference lines, while *EXP1* is induced in *SlLOB1*-overexpressing transgenic leaves and fruit. In contrast to the role of ethylene and previously characterized ripening TFs, which are comprehensive facilitators of ripening phenomena including softening, *SlLOB1* participates in a regulatory subcircuit predominant to cell wall dynamics and softening.

transcription factor | SlLOB1 | softening | cell wall | ripening

Tomato (*Solanum lycopersicum*) is a widely studied model of fleshy fruit development and ripening (1, 2). Softening is an important aspect of ripening physiology, as it determines palatability for frugivores and is a key factor in determining damage and loss in fruit food supply chains. Genotypes inhibited in ripening, early harvest, and controlled atmospheres limiting respiration and ethylene synthesis are deployed to maintain firmness and shelf life, often at the expense of quality. A clearer understanding of the genetic basis of fruit softening and ripening regulation are essential to optimize shelf life and quality for food and nutritional security.

Ripening-related textural changes are closely associated with cell wall metabolism, and extensive efforts have focused on understanding tomato fruit cell wall remodeling and the underlying genes (3, 4). Particularly notable wall modifications during ripening include depolymerization of pectins and hemicelluloses and pectin solubilization, which contribute to dissolution of the middle lamella, reduced cell adhesion, and cell wall swelling (3, 4). These are orchestrated by an array of cell wall–modifying proteins, the most studied of which is endo-polygalacturonase 2a (*PG2a*) from tomato. *PG2a* is encoded by a fruit-specific and ripening-induced gene that is responsible for up to 1% of messenger RNA (mRNA) in ripening pericarp (5), and its repression was the basis of the first commercialized transgenic plant, Flavr Savr (6). Numerous additional cell wall–degrading enzymes have been characterized, including pectate lyase (*PL*) pectin methylesterase (*PE2*, *Pmeu1*), β-galactosidase (*TBG4*) (3, 4), endoglucanase (*CEL2*) (7), and xyloglucan endotransglucosylase/hydrolases (*XTH5*) (8), also homologs from other fruit species (9). Additionally, expansin proteins [e.g., *EXP1* 10], which have no known enzymatic activity, contribute to cell wall loosening and textural changes (9, 10). However, altering the expression of these genes individually does not have substantive effects on softening with the notable exception of *PL* (11). Thus, a deeper understanding of fruit textural changes requires examination of higher order regulators that influence multiple cell wall–related genes.

Tomato-ripening mutants such as *rin* (*ripening inhibitor*, encoding a MADS-box transcription factor [TF]), *Cnr* (*Colorless nonripening*, encoding an SBP-box TF), and *nor* (*nonripening*, encoding a NAC TF) inhibit softening in addition to many other ripening characteristics, including color, flavor, ethylene hormone synthesis, and aroma (12–14). While the *rin* mutation has been shown to have dominant gain-of-function repressor action, the *RIN* gene is nevertheless critical in virtually all ripening activities (15, 16). Additional ripening genes have been identified through mutations or gene expression profiles and some functionally characterized, including the TF genes *TAGL1*, *FUL1*, *FUL2*, *MADS1*, *NAC1*, *AP2a*, and *SlGRAS38* (1, 2, 17, 18). As with *RIN*, these influence a broad range of ripening processes, such as ethylene synthesis, pigmentation, time to initiation, and completion of ripening, and many cell wall–associated genes (e.g., *PG2a*, *EXP1*, *PL*, *PE2*, and *TBG4*) have altered expression in mutant or repression lines of

Significance

A tomato fruit ripening–specific transcription factor, *SlLOB1* predominantly influences fruit cell wall–related gene regulation and textural changes during fruit maturation and thus is distinct from broadly acting ripening transcription factors described to date that influence many ripening processes. As such, *SlLOB1* is an intermediate regulator primarily influencing a physiological subdomain of the overall ripening transition.

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these TFs (1, 2, 17–19). Two additional TFs, *EREBP6* and *LeHB1*, are more specific to ethylene synthesis (1, 2). In the case of *RIN*, many are direct targets (20). Importantly, however, TFs that primarily target only the cell wall–modifying component of the ripening cascade have yet to be reported.

Earlier molecular studies revealed the genetic basis of ethylene synthesis (21, 22) and responses (23) and highlighted the essential role of this gaseous hormone in ripening phenotypes, including softening. Ethylene is a coordinator of ripening pathways acting in concert with many of the ripening TFs. Ethylene response factor (*ERF*) genes are TFs that reside at the end of the ethylene signaling pathway, and several tomato fruit ripening–and softening–related *ERFs* have been described. For example, *AP2a* RNA interference (RNAi) fruit are softer due to enhanced ethylene production, suggesting a negative regulatory effect (17). Additionally, overexpression of tomato *LeERF1* was reported to accelerate ripening, including softening, while its repression extended shelf life (24). *ERF2.2* underlies a firmness quantitative trait loci (*Firs.p.QTL2.2*) but has not yet been functionally characterized (25).

We searched public tomato fruit transcriptome data [https://tea.solgenomics.net (18, 26)] for TFs induced both in ripening pericarp and just prior to ripening in the locular gel surrounding the seeds. We hypothesized that genes expressed in the solubilizing locular gel immediately before ripening and in the pericarp at ripening initiation might participate more exclusively in cell wall–related activities. A tomato LATERAL ORGAN BOUNDRIES (LOB) domain gene, *SlLOB1*, matched this profile. *LOB* genes belong to a plant-specific TF family of 42 members in *Arabidopsis thaliana* and 35 in rice (*Oryza sativa*) (27). Based on the structure of the N terminus LOB domain, two subfamilies have been defined. Class I LOB proteins contain a complete LOB domain comprised of three conserved subdomains: the C domain involved in DNA binding, the GAS (Gly-Ala-Ser) domain, and the L domain for protein interaction. Class II LOB proteins lack the L domain (28–30). Most *LOB*s belong to the class I subfamily, including *SlLOB1*. LOB proteins have an essential role in lateral organ development, including lateral organ initiation and patterning, pollen and root development, plant regeneration capacity, pathogen responses, and secondary xylem and phloem growth in addition to metabolic process (anthocyanin and nitrogen metabolism) (28), while one has been associated with ripening banana fruit and expansin gene expression (31). Using the reference tomato genome sequence, 46 tomato *LOB*s were identified (32), and only one member, *SlLBD40*, has been functionally defined to date with evidence suggesting a role in drought tolerance (33).

Using RNAi repression and ectopic expression in transgenic tomato plants, we demonstrate that *SlLOB1* acts as a transcriptional activator of a broad suite of cell wall–related genes and fruit softening. Additional ripening phenotypes were minimally affected including ripening initiation, onset of the ethylene burst, and full ripe fruit appearance, although elevated carotenoid levels were observed in ripe fruit of the repressed lines. In contrast to many previously described *LOB* genes, *SlLOB1* repression revealed no significant phenotypes associated with organ development or differentiation, though such phenotypes were observed with ectopic overexpression. *SlLOB1* is a ripening-related TF that is distinct from those described to date in that its primary targets are a suite of genes mediating cell wall and textural changes, a distinct subset of the late fruit development and ripening program.

Results

Tomato *SlLOB1* Is Predominantly Expressed in Maturing Fruit. *SlLOB1* expression in the pericarp coincides with ripening. *SlLOB1* is initially induced at the mature green (MG) stage, highly expressed at early ripening (breaker stage [BR]), and drops slightly after BR. Interestingly, in the MG locule, which becomes liquefied prior to pericarp ripening, *SlLOB1* mRNA accumulates at three times the rate in pericarp (Fig. 1*A*). Expression of *SlLOB1* occurred minimally in vegetative and floral tissues (Fig. 1*A*). Stems have the highest nonfruit expression yet less than 10% of levels in the immature green (IMG) locule. Locular gel expression is higher than in pericarp at all stages except BR in which levels are similar (Fig. 1*A*). Initial *SlLOB1* expression in IMG locule is sixfold that of IMG pericarp. Notably, the *RIN*, *NOR*, and *DML2* ripening regulator genes have been reported to show similar expression profiles (2).

***SlLOB1* Repression Results in Reduced Softening and Extended Shelf Life.** To investigate the function of *SlLOB1* in fruit ripening, we generated seven independent *SlLOB1* RNAi tomato lines, three of which (#1, #3, and #6) were assessed in the T1 generation. T2 generations were developed for the two mostly strongly repressed lines, #3 and #6 (*SI Appendix*, Fig. S1). *SlLOB1* RNAi lines showed normal growth phenotypes as compared to wild type (WT), with no notable differences observed (*SI Appendix*, Fig. S2) and consistent with its expression being predominant in maturing fruit tissues. *SlLOB1* mRNA levels in T2 RNAi BR pericarp and locule tissues were observed to be reduced to ≤10% of levels in untransformed WT (Fig. 1*B*).

To evaluate fruit softening, we measured both whole fruit compression and pericarp penetration force using a texture analyzer.

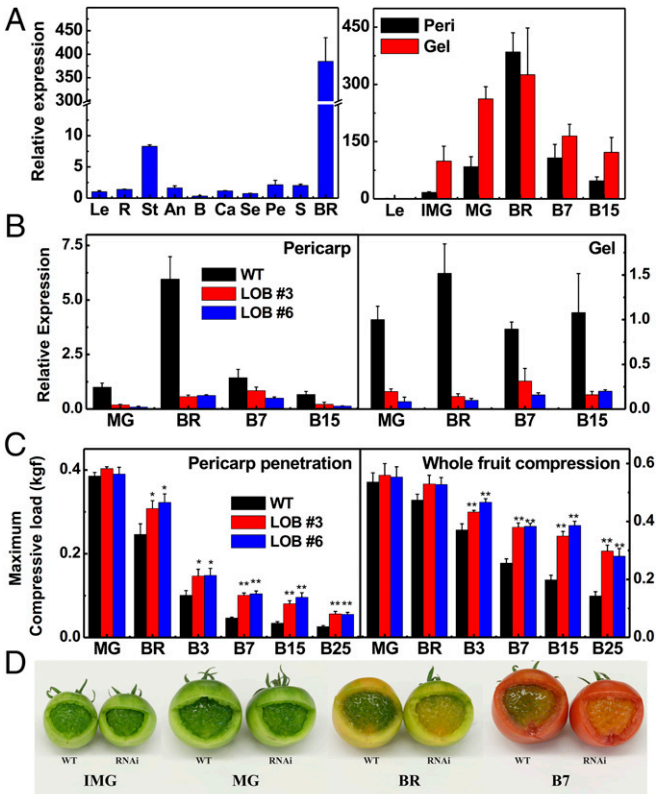


Fig. 1. Fruit gene expression and phenotypes following *SlLOB1* repression. (*A*) Expression of *SlLOB1* in WT tissues leaves (Le), root (R), and stem (St) of 2-wk-old seedlings, anthers (An), floral buds (B), prepollination carpel (Ca), sepals (Se), petals (Pe), seed (S), breaker stage (BR), immature green (IMG), mature green (MG), breaker + 7 d (B7), and breaker + 15 d (B15). (*B*) Relative transcript abundance of *SlLOB1* in WT and two RNAi lines (LOB #3 and #6) at stages MG, BR, B7, and B15 of pericarp and locule (gel). (*C*) Fruit firmness measured by fruit compression and pericarp penetration at the indicated developmental stages. $*P < 0.05$, $**P < 0.01$. (*D*) WT and *SlLOB1* RNAi (LOB #3) fruit at indicated developmental stages (see *A*) with pericarp partially removed to see locule development. Error bars indicate SE.

Increased firmness was evident from BR through the red-ripe (RR) stage (Fig. 1*C*). Pericarp penetration force was the same in transgenic fruit as in WT at MG but was 25% higher at BR, and more than twice the force was needed at BR + 7 d (B7) and BR + 15 d (B15). A similar trend was observed for whole fruit compression (Fig. 1*C*). *SlLOB1*-repressed fruit displayed reduced collapse after 30 d of storage, although they were similar to WT after 60 d (Fig. 2*A*). Water loss from the fruit during storage, determined gravimetrically, was also substantially lower in the transgenic fruit (Fig. 2*B*). We also measured the fruit cuticle thickness (*SI Appendix*, Fig. S3*A*) and the force needed to penetrate the cuticle (*SI Appendix*, Fig. S3*B*), but neither showed a difference between genotypes, suggesting no major biomechanical role for the cuticle in the enhanced firmness phenotype of the *SlLOB1* RNAi fruit.

SlLOB1-repressed fruit displayed less locule liquefaction (conversion of the locule tissue to a liquid or jelly-like state of normally ripe tomato fruit) than WT fruit at the same stage (Fig. 1*D*), and, similar to the prior description of fruit of the *Cnr* mutant (13), *SlLOB1*-repressed locule tissue also released water more readily than that from WT (*SI Appendix*, Fig. S4 *A* and *B*), similar to observations for the *Cnr* mutant (13). *Cnr* cell wall extracts do not swell when hydrated, reflecting their more intact structure (34), and repression of *SlLOB1* had a similar effect, with swelling reduced by 20% compared to WT (*SI Appendix*, Fig. S4*C*). In addition, *SlLOB1*-repressed cell wall extracts from the locular gel showed a similar pattern of precipitation to those from *Cnr*, which was distinct from WT extracts (*SI Appendix*, Fig. S4*B*). We noted that, while *Cnr* fruit float in water, this was generally not the case in the repression lines. However, it was observed in fruit from a single overexpression line (*SI Appendix*, Fig. S4*D*) that showed the greatest repression of most cell wall genes (*SI Appendix*, Table S1) and greater fruit firmness than the RNAi fruit, presumably due to cosuppression (*SI Appendix*, Fig. S5). Moreover, *SlLOB1* RNAi fruit floated in 3.6% sucrose solution while those from WT sank, indicating reduced density (*SI Appendix*, Fig. S4*E*). While the function of the CNR gene has recently been questioned (46), the *Cnr* mutant displays many attributes of fruit with altered cell walls (13). Our comparison to *Cnr* reinforces that *SlLOB1* repression is consistent with extensive cell wall alterations but does not say anything regarding *CNR* gene function.

Identification of Cell Wall Genes Influenced by *SlLOB1* Suppression. We performed RNA sequencing (RNA-seq) transcriptome analysis of B7 pericarp and locule tissues from WT and two T1 repression lines (Datasets S1 and S2 and Fig. 3 *A*–*C*). Consistent with the phenotypic strength (*SI Appendix*, Fig. S1), line #6 had more DEGs (differentially expressed genes, cutoff: $P < 0.05$; ration > 2 or < 0.5) than line #3. In addition, the pericarp had more DEGs than locule tissue (Fig. 3 *A*–*C*). A gene ontology (GO) analysis of down-regulated DEGs revealed enrichment in cell wall–related transcripts (*SI Appendix*, Table S2). To identify genes with the strongest support for influence by SlLOB1, we focused on those with differential expression in both tissues. A total of 34 genes were commonly down-regulated in all four comparisons of pericarp and locular gel from the two RNAi lines compared to WT (Fig. 3*B*). Of these, 10 are related to cell wall modification (Fig. 3*D*), including expansin, endo-1,4-β-glucanase, xylosidase, pectate lyase, and mannanase. Additional putative cell wall genes that were differentially expressed in either pericarp or locule tissues are listed (*SI Appendix*, Fig. S6*A*). Notably, *PG2a* was up-regulated in *SlLOB1* RNAi lines (*SI Appendix*, Fig. S6*A*), though we note prior investigations indicate a minimal role of this enzyme in tomato softening (3, 4, 36, 37). *PL* was not altered in pericarp but was repressed in the locule of *SlLOB1* RNAi fruit (line #6) (*SI Appendix*, Fig. S6*B*).

Based on GO term enrichment, 47 TFs were down-regulated and five were up-regulated in fruit from both *SlLOB1*-repressed lines (*SI Appendix*, Table S2). *SlLOB1* was the only differentially expressed *LOB*, supporting the specificity of RNAi-mediated gene suppression (*SI Appendix*, Fig. S7*A*). Among the down-regulated TFs, members of the *zf-RING, F-box*, and *bHLH* families accounted for 39% of the total (*SI Appendix*, Fig. S7*A*). Several TFs displayed substantial down-regulation in both locular gel and pericarp, including the *HD-Zip* genes *SlANL2b* (Solyc06g035940.2) and Solyc03g120910.2 as well as *BSD* (BTF2-like transcription factors, Synapse-Associated, and DOS2-like proteins) (Solyc07g022920.2), *MYB-like* (Solyc06g066340.2), *WOX* (Solyc02g082670.2), and

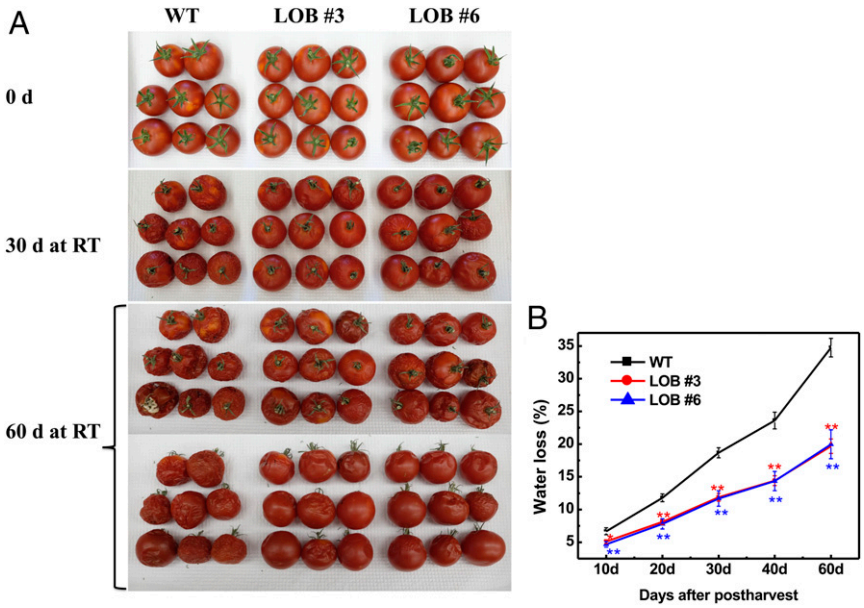
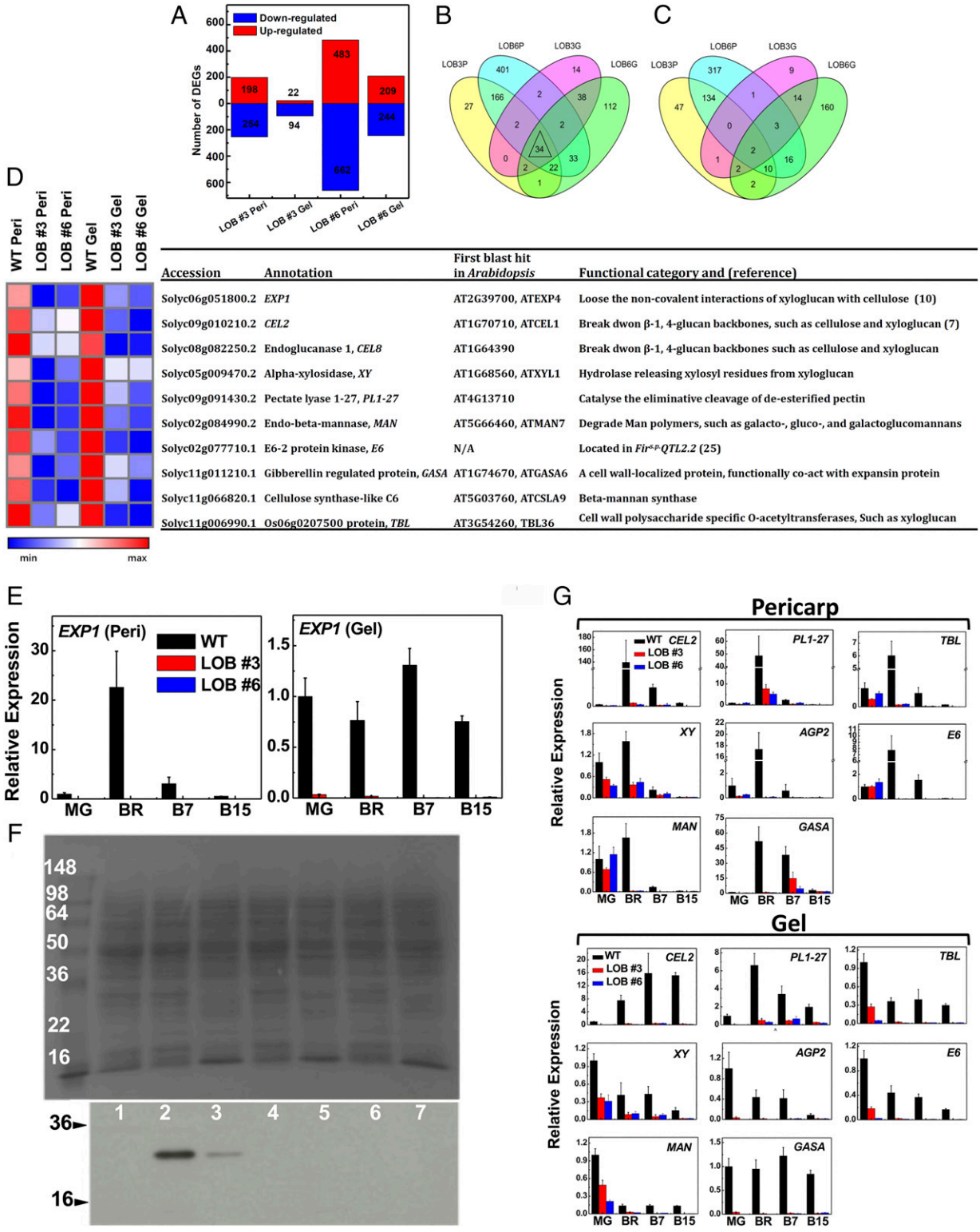


Fig. 2. Postharvest shelf life and water loss of T2 *SlLOB1* RNAi fruit. (*A*) WT and LOB #3 and #6 fruit were harvested at B15 and stored at room temperature (25 °C) and photographed at the indicated days postharvest. (*B*) The same fruit shown in *A* were weighed at the indicated days postharvest to measure water loss (**$P < 0.01$). Error bars indicate SE.



Accession	Annotation	First blast hit in *Arabidopsis*	Functional category and (reference)
Solyc06g051800.2	*EXP1*	AT2G39700, ATEXP4	Loose the non-covalent interactions of xyloglucan with cellulose (10)
Solyc09g010210.2	*CEL2*	AT1G70710, ATCEL1	Break dwon β-1, 4-glucan backbones, such as cellulose and xyloglucan (7)
Solyc08g082250.2	Endoglucanase 1, *CEL8*	AT1G64390	Break dwon β-1, 4-glucan backbones such as cellulose and xyloglucan
Solyc05g009470.2	Alpha-xylosidase, *XY*	AT1G68560, ATXYL1	Hydrolase releasing xylosyl residues from xyloglucan
Solyc09g091430.2	Pectate lyase 1-27, *PL1-27*	AT4G13710	Catalyse the eliminative cleavage of de-esterified pectin
Solyc02g084990.2	Endo-beta-mannase, *MAN*	AT5G66460, ATMAN7	Degrade Man polymers, such as galacto-, gluco-, and galactoglucomannans
Solyc02g077710.1	E6-2 protein kinase, *E6*	N/A	Located in *Fir*$^{s.p}$ *QTL2.2* (25)
Solyc11g011210.1	Gibberellin regulated protein, *GASA*	AT1G74670, ATGASA6	A cell wall-localized protein, functionally co-act with expansin protein
Solyc11g066820.1	Cellulose synthase-like C6	AT5G03760, ATCSLA9	Beta-mannan synthase
Solyc11g006990.1	Os06g0207500 protein, *TBL*	AT3G54260, TBL36	Cell wall polysaccharide specific O-acetyltransferases, Such as xyloglucan

Fig. 3. Transcriptome analysis, cell wall gene expression, and EXP1 protein of *SlLOB1* RNAi lines. (*A*) Overview of DEGs in *SlLOB1* repression lines. (*B*) Venn diagram of overlapping down-regulated genes between *SlLOB1* repression lines LOB #3 and LOB #6 pericarp and locule (gel) compared to WT. (*C*) Venn diagram of overlapping up-regulated genes of tissues as in *B*. (*D*) Subset of the 10 cell wall–associated DEGs in *B*, annotation, functional categories, and expression (log 2 of reads per kilobase million). (*E*) Time course qRT-PCR of *EXP1* in pericarp and locule (gel) of WT and *SlLOB1* RNAi fruit. MG, BR, B7, and B15 tissues were compared to MG WT as reference (defined as 1). (*F*) Detection of EXP1 protein in *SlLOB1* RNAi and control Breaker fruit. (*Upper*) Total protein ponceau staining. (*Bottom*) Immunoblotting. 1, WT MG pericarp (peri); 2, WT BR peri; 3, WT BR locule (gel); 4, LOB #3 BR peri; 5, LOB #3 BR gel; 6, LOB #6 BR peri; 7, LOB #6 BR gel. (*G*) qRT-PCR validation of selected cell wall genes at MG, BR, B7, and B15 with WT MG used as reference (defined as 1). Error bars indicate SE.

zf-C2H2 (Solyc06g062670.2) (*SI Appendix*, Fig. S7*A*). *SlANL2b* (Solyc06g035940.2) is paralogous to *CD2* (*CUTIN DEFICIENT 2*), a regulator of epidermal cell cuticle deposition (38) whose orthologous *A. thaliana* loss-of-function mutant increased cell wall polysaccharide content (39). *Solyc03g120910.2* is orthologous to an *A. thaliana* gene involved in vascular development and parenchyma pith cell primary wall retention (40). *SlGRAS38* (Solyc07g052960.1), a target of RIN and also a regulator of broad ripening phenomena including time to ripening initiation, was repressed in *SlLOB1* repression fruit (*SI Appendix*, Fig. S7*A*) (18), while other functionally identified ripening TFs were not substantially influenced by *SlLOB1* (*SI Appendix*, Fig. S7*B*).

Validation of Cell Wall Gene Repression. Nine cell wall–related DEGs were selected for qRT-PCR validation in T2 generation RNAi fruit, and differential expression was confirmed in each case. *EXP1* displayed the most substantial down-regulation (<1.5% of WT in pericarp and <3.3% in locule) (Fig. 3*E*). Notably, this degree of repression is greater than that observed in antisense *EXP1* fruit (10). Immunoblot analysis with an EXP1 antibody detected the predicted 25 kDa protein in protein extracts from WT but not transgenic fruit (Fig. 3*F*). *CEL2* (7), which encodes an endo-β-1,4-glucanase, was down-regulated in all four stages of pericarp and locule to as little as 5 and 3% of WT in lines #3 and #6 locular gel, respectively. Other repressed genes were *XY* (alpha-xylosidase), *MAN* (beta-1,4-endomannase), and *PL1-27* (pectate lyase), three predicted cell wall–metabolizing enzymes with potential roles in xyloglucan side chain modification, galactomannan backbone hydrolysis, and homogalacturonan breakdown, respectively. Additionally, *AGP2*, a cell wall glycoprotein (35); *E6*, which is a candidate for firmness *QTL2.2* (25); *GASA* (Gibberellic Acid-stimulated Arabidopsis), which has been shown to influence cell expansion in *A. thaliana* (41); and *TBL* (Trichome Birefringence-Like), involved in *O*-acetylation of hemicelluloses and pectic polysaccharides (42), were all down-regulated in *SlLOB1*-repressed fruit (Fig. 3*G*).

Ectopic Expression of *SlLOB1* Promotes Softening. We generated transgenic *SlLOB1* ectopic expression plants; and six independent tomato lines were recovered, although three (OE1, OE5, and OE12) proved to be cosuppression lines, and only OE1 was further characterized in the context of gene suppression (*SI Appendix*, Figs. S4*D*, S5, and S8). The remaining three overexpression lines (OE2, OE6, and OE13) displayed similar pleiotropic phenotypes, including increased branching, dwarfism, enlarged pedicels, and reduced internode length (measured in the first three trusses), all consistent with altered organ boundary formation, though no discernable changes in leaf architecture were noted, and smaller fruit resulted with enlarged pedicel and seeds with abnormal seeds coats (Figs. S2 and S9 *A*–*D*). *SlLOB1* RNAi fruit yielded seed of normal appearance (*SI Appendix*, Fig. S9*E*) and number that underwent normal germination, consistent with the low expression of *SlLOB1* in seed [https://tea.solgenomics.net (18)]. Moreover, fruit became soft and the locule liquefied prior to ripening consistent with the reduced softening of *SlLOB1* RNAi repression (Fig. 4 *A*–*C* and *SI Appendix*, Fig. S10).

A sufficient number of fruit for softening analyses were only available from lines OE2 and OE6. IMG of *SlLOB1* OE locule liquefied prematurely (Fig. 4*A*). Additionally, both whole fruit compression and pericarp penetration assays showed that OE fruit were softer that WT fruit, with measurable differences detected as early as the IMG stage (Fig. 4 *B* and *C* and *SI Appendix*, Fig. S10). The firmness of the OE2 pericarp was 50% of WT at BR, though WT fruit rapidly softened as they matured, such that there was no significant difference at the B7 stage between transgenic and WT fuit (Fig. 4 *B* and *C* and *SI Appendix*, Fig. S10). Following harvest, OE fruit showed overt signs of water loss (wrinkling) in advance of WT (*SI Appendix*, Fig. S11*A*) and substantially greater water loss:

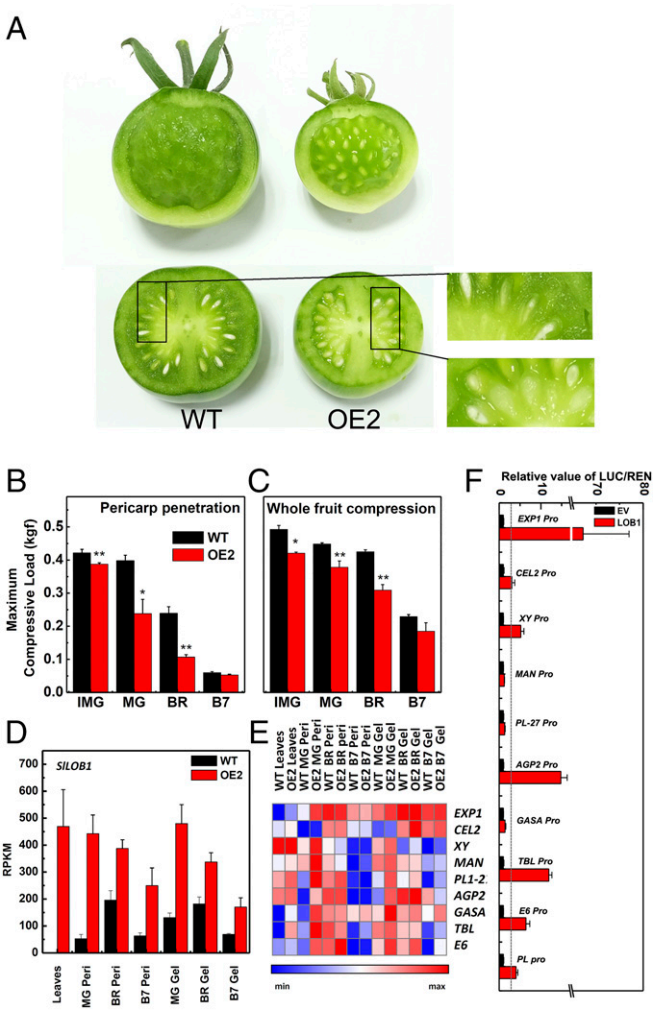


Fig. 4. Effects of *SlLOB1* overexpression fruit. (*A*) Premature locule liquefaction occurs in locule of *SlLOB1* overexpression fruit. WT (*Left*) and *SlLOB1* OE2 (*Right*). (*B*) Softening as defined by pericarp penetration. (*C*) Softening as defined by whole fruit compression (*$P < 0.05$, **$P < 0.01$). (*D*) *SlLOB1* expression in *35S: LOB1 OE2* and WT fruit tissues. (*E*) Heat map of cell wall gene expression in *35S: LOB1* OE2 leaves and fruit (log10 of reads per kilobase million [RPKM]). Error bars indicate SE. (*F*) Dual luciferase assay. EV indicates empty vector with output defined as 1. The dotted line indicates LUC/REN = 3. Error bars indicate SE.

26% weight loss from OE6 fruit after 20 d storage, compared with 11% from WT (*SI Appendix*, Fig. S11*B*).

Transcriptome analysis (Dataset S3) of OE2 tissue showed that the *SlLOB1* transcript in OE2 leaves was 357-fold greater than WT, and expression in OE2 MG pericarp was 19-fold that measured in WT (Fig. 4*D*). *SlLOB1* mRNA accumulation increased 2.4-fold in the OE2 MG locule, consistent with its high endogenous expression. Modest increases in *SlLOB1* expression were also observed in ripening fruit tissues (Fig. 4*D*), again consistent with high endogenous gene expression. We also checked the expression of nine cell wall genes in OE2 young leaves and fruit. In leaves, *EXP1*, *TBL*, and *GASA* transcripts were higher than in WT, with the others showing minimal differences. In fruit tissues, transcript accumulation of all nine genes was elevated in parallel with the relative increase in *SlLOB1* expression (Fig. 4*E*). This was confirmed by qRT-PCR in OE6 (*SI Appendix*, Fig. S12). *PL* was induced to 5.5-fold in OE2 MG pericarp (*SI Appendix*, Fig. S6*B*). These results demonstrate that SlLOB1 is sufficient to induce expression in leaves for some genes, but for others, additional

factors may be limiting. Finally, cell wall antibody probes were used to assess changes in cell wall polysaccharides and revealed reduced xyloglucan and homogalacturonan levels in BR stage *SlLOB1* overexpression pericarp as compared to WT (*SI Appendix*, Fig. S13).

SlLOB1 Activates *EXP1*, *CEL2*, *XY*, *AGP2*, *TBL*, *E6*, and *PL* Promoters In Vitro. To better place *SlLOB1* function in the context of other *LOB* genes, a phylogenetic analysis of *LOB1* orthologs was performed (*SI Appendix*, Fig. S14). *LOB1* and *LOB11* family members are close homologs in many species. *A. thaliana* AtLBD1 and AtLBD11 have 77% amino acid identity, while tomato SlLOB1 and SlLOB11 share 82% identity. *SlLOB11* is mainly expressed in young roots and only minimally in fruit (*SI Appendix*, Fig. S14). *AtLBD1* is functionally undefined, though citrus (*Citrus sinensis*) and poplar (*Populus tremula* × *Populus alba*) orthologs, *CsLOB1* and *PtaLBD1*, participate in citrus bacterial canker susceptibility and secondary wood formation, respectively (43, 44).

SlLOB1 contains conserved domains, consistent with DNA binding and protein–protein interactions (*SI Appendix*, Fig. S14). LOB family members can bind the promoter and activate *EXP* gene expression in *A. thaliana* and banana (28, 29, 31). To better understand *SlLOB1* function, we carried out promoter transactivation using transient expression in *Nicotiana benthamiana*. This revealed that SlLOB1 has activator activity on the *EXP1*, *CEL2*, *XY*, *AGP2*, *TBL*, *E6*, and *PL* promoters as defined by greater than threefold activity compared to controls (Fig. 4*F*). *EXP1* transactivation was especially strong (67-fold induction). No SlLOB1 activation was observed for the *MAN*, *PL1-27*, and *GASA* promoters (Fig. 4*F*).

The abundance of *EXP1* mRNA in response to ectopic *SlLOB1* transgene expression and stronger promoter induction in the transactivation assay and substantial reduction of transcript and protein levels in *SlLOB1* repression lines was particularly notable. To identify *cis* elements in the *EXP1* promoter that bind SlLOB1, we generated an *EXP1* promoter deletion series. These deletions, designated P1 to P5, were used to develop luciferase reporter constructs (*SI Appendix*, Fig. S15*A*). Promoter activity decreased to 72% of the full-length promoter activity in the first deletion (P1_−1,393 base pair [bp] to P2_−1,094 bp), while subsequent deletions (comparing P2 to P4 and P4 to P5) had little effect. With deletion P5, reporter activity decreased to only eightfold induction, suggesting at least two regulatory loci at −1,393~ −1,094 and −389~0. P3 (−1,094~ −568) were not activated by SlLOB1 as predicted. Using the *cis* element prediction websites, PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and Softberry (http://www.softberry.com/), we identified two putative *cis* elements: I (ACCTCAAAT) and II (ATTTTCTTCA), based on their presence in both regions (*SI Appendix*, Fig. S15*A*). We next generated deletion P6, missing predicted *cis* element I but carrying II, as well as deletion P7, with I but without element II. P7 showed only threefold activation, while P6 had no effect, indicating that SlLOB1 is functional without element I (*SI Appendix*, Fig. S15*A*).

Silencing *SlLOB1* Altered Carotenoids but Not Additional Fruit-Ripening Phenotypes. Suppression of *SlLOB1* expression had profound effects on the cell wall–associated transcriptome, softening, fruit water loss, and shelf life but did not affect ripening initiation as measured in time to the BR stage following anthesis (*SI Appendix*, Fig. S16*A*). Ethylene production by the transgenic fruit was not different from the WT fruit in terms of time of initiation or stage of maximal production but remained higher during later fruit development when *SlLOB1* was repressed (*SI Appendix*, Fig. S16*A*). The characterization of *ACS* and *ACO* ethylene synthesis genes in ripening fruit of WT and *SlLOB1*-repressed lines (*SI Appendix*, Fig. S16*B*) indicated fluctuation in several family members but nothing that could be readily interpreted as the molecular basis for elevated ethylene in later stage RNAi fruit. The repression of *SlLOB1* resulted in noticeably darker red fruit, especially in the locular gel (*SI Appendix*, Fig. S16*C*). To better understand the basis of this color change, we characterized carotenoid profiles in both locule and pericarp tissue from B7 and B15 stage fruit by high-pressure liquid chromatography (HPLC) analysis. In the gel, lycopene and beta-carotene levels were approximately twice those in WT at both stages, while in pericarp, lycopene abundance was ~60 and 40% higher in B7 and B15 fruit, respectively. No differences were noted in beta-carotene levels (*SI Appendix*, Fig. S16*D*).

Discussion

***SlLOB1* Regulates Multiple Fruit Cell Wall–Associated Genes and Softening.** In addition to altering expression of genes encoding cell wall proteins, orthologs of *A. thaliana* cell wall–related TFs (e.g., Solyc06g0359400.2 and Solyc03g1209100.2) responded to *SlLOB1* repression in fruit. Ectopic expression of *SlLOB1* activated the same genes prematurely in green fruit, leading to precocious textural changes and softening. Together, these genes are associated with the modification of all three major cell wall polysaccharide classes (cellulose, pectins, and hemicellulose) in addition to cell wall glycoproteins. The corresponding synergistic activities, mediated through a single regulator, manifest in more substantive textural changes than previously observed when targeting a single cell wall–associated gene. It is noteworthy that the gene shown to exert the largest effect on softening to date, *PL* (11), is positively regulated by SlLOB1, and its promoter can be activated by SlLOB1 in vitro.

***EXP1* Is a Direct Target of SlLOB1, and Additional *EXP* Genes Are Influenced by *SlLOB1* Repression.** *EXP1* is the most altered DEG by *SlLOB1* manipulation. Expansins are widely studied in fleshy fruits (9, 10) with induction paralleling softening in all cases. It has been proposed that EXP disrupts noncovalent interactions between cellulose microfibrils and matrix polysaccharides, facilitating access to cell wall–modifying enzymes, and thus may be especially important to softening in the context of coregulated cell wall catalytic activities (45).

Similar to tomato SlLOB1 and *EXP1*, *A. thaliana* AtLBD18 binds to the *AtEXP14* promoter, though in this case, it promotes lateral root emergence (29). CsLOB1 was observed to induce expansin expression following transient overexpression in sweet orange leaves (43), and banana *MaLBD1/2/3* was reported to induce expansin promoter activity in tobacco BY-2 protoplasts (31). It is noteworthy that in addition to *LeEXP1* (Fig. 3*E*), two additional *EXP* homologs (Solyc01g090810 and Solyc08g077910) were downregulated in *SlLOB1* repression lines (*SI Appendix*, Fig. S6*A*).

Gene Expression and Phenotypes Suggests *SlLOB1* Acts Downstream of More Global Ripening Regulators. The *Cnr* epiallele results in impaired ripening, reduced ethylene synthesis, extensive cell wall modification, and softening inhibition (13), although the degree of ripening inhibition has recently been questioned (46). *SlLOB1* RNAi-repressed fruit are characterized by *Cnr*-like texture in that they are firmer and have less locule solubilization, reduced cell wall swelling, and decreased fruit density (*SI Appendix*, Fig. S4). *SlLOB1* expression is reduced over fourfold in the *Cnr* mutant (*Cnr* 42DPA/WT 42DPA = 0.223, P value = 0.01), and pericarp DEGs of suppression line 6 (B7 pericarp) compared to published *Cnr* data [BR pericarp (20)] indicated 99 common down-regulated and 49 up-regulated genes. A total of 11 of the common down-regulated genes are cell wall associated (Dataset S4). The *SlLOB1* promoter contains the GTAC binding motif of SPB proteins (47). However, the *SlLOB1* promoter was not activated by CNR in a transient expression system (*SI Appendix*, Fig. S17). The similar firmness, locule liquefaction, fruit density, and cell wall swelling phenotypes of *Cnr* and *SlLOB1* repression

fruit suggest consequences of some, or all, of the common cell wall–associated DEGs operating together.

The results here demonstrate that *SlLOB1* effects are targeted primarily to the fruit locule and pericarp cell wall and textural changes and, as such, affect ripening downstream of more global ripening regulators such as *RIN*. Indeed, *SlLOB1* is repressed in both *rin* and *nor* mutants (20, 46). TFs encoded by Solyc04g081190_*bZIP* and Solyc06g035940.2_*HD-Zip* genes are also induced by SlLOB1 (*SI Appendix*, Figs. S7*A* and S17), indicating *SlLOB1* operates in part via additional downstream TFs. Such genes might be responsible for altered expression of genes differentially expressed in response to *SlLOB1* repression but whose promoters do not interact with SlLOB1. It is noteworthy that *LOB* genes have an intermediate placement in regulatory networks defined in other species. For example, in *A. thaliana*, LOBs can regulate *AP2*, *WOX*, or *E2Fa* positively and *KNOX* negatively, while BZR1, ARF, and NAC consensus binding sites are located upstream of different *LOBs* (28). Together, these interactions suggest that *SlLOB1* operates downstream of more comprehensive ripening regulators (e.g., *RIN* and *NOR*) but upstream of other regulators, such as *bZIP* and the *HD-Zip* genes noted. Furthermore, members of several TF families, including MYB, bHLH, and LOB itself, are candidates for direct SlLOB1 interaction partners, as members of these families are known from studies of *A. thaliana* to form dimer or trimers with LOB proteins (28, 48). In these cases, they integrate regulatory connections between primary transcriptional regulators and downstream outputs (*SI Appendix*, Fig. S15*B*). Finally, a single tomato TF, *SlMBP3*, a member of the AGAMOUS subfamily of tomato MADS-box genes, was recently shown to be necessary for tomato seed development and locule liquefaction (49). *SlMBP3* expression is predominant in the seed at anthesis and early development, strongly expressed in locular tissue postanthesis and through fruit development with much lower expression in carpel tissues [https://tea.solgenomics.net (18)]. The repression of *SlMBP3* resulted in similar inhibition of locule liquefaction as with *SlLOB1* repression reported here (Fig. 1*D*) but with additional phenotypes of altered seed coat development, reduced seed viability, and substantially reduced fruit size (49). Together, these results suggest *SlLOB1* is more specific to locule liquefaction, while *SlMBP3* has broader pleiotropic effects on fruit development. *SlLOB1* presents a genetic target to more precisely modify locule liquefaction and texture absent effects on seed viability and fruit size.

***SlLOB1* Influences Ethylene Production in Later Ripening in Addition to Carotenoid Profiles.** The repression of *SlLOB1* had no effect on ethylene production during early ripening when fruit ethylene is at its highest, and the cascade of changes summing to render the ripe phenotype are initiated. As the fruit continues ripening, ethylene decreases, and this decrease was attenuated in *SlLOB1* repression fruit (*SI Appendix*, Fig. S16*A*). Fruit overexpressing *SlLOB1* began softening well before their WT counterparts prior to the induction of ripening ethylene (Fig. 4*B* and *SI Appendix*, Figs. S10 and S18). While prior data indicate a clear necessity for ethylene in tomato fruit softening (21, 22), the data presented here indicate that *SlLOB1* is a more immediate effector of mature fruit textural changes through the activation of multiple cell wall–associated genes.

Unlike ethylene synthesis–inhibited tomato fruit or the ripening-repressed *Cnr* and *rin* mutant fruit, *SlLOB1* RNAi fruit accumulate carotenoid pigments, although ultimately, they accumulate higher levels of lycopene and β-carotene than nontransgenic controls. Expression of carotenoid enzymatic genes was somewhat higher in both the pericarp and locule of the *SlLOB1*-repressed fruit, particularly for the rate-limiting step of phytoene synthase conferred by the *PSY1* gene, consistent with enhanced carotenoid accumulation in both the pericarp and locule tissues (*SI Appendix*, Fig. S19). The fact that *PSY1* is ethylene inducible (1) and most elevated in later stage fruit (*SI Appendix*, Fig. S19) suggests that the elevated carotenoid phenotype may be related to the elevated ethylene (*SI Appendix*, Fig. S16*A*). SlLOB1 did not interact directly with the *PSY1* or *PDS* promoters in an in vitro activation assay (*SI Appendix*, Fig. S17). *SlLOB1* ectopic expression resulted in reduced pericarp carotenoid accumulation but enhanced lycopene accumulation in locular gel, which is consistent in all lines (*SI Appendix*, Fig. S20 *A* and *B*). Additionally, five heat shock proteins (HSPs) were strongly induced in both *SlLOB1* RNAi lines (*SI Appendix*, Fig. S21), including *HSP21*, which has previously been associated with lycopene accumulation (50).

In conclusion, our results demonstrate that *SlLOB1* functions as a positive transcriptional regulator of fruit softening through its activities in both the locule and pericarp. The repression of *SlLOB1* inhibits fruit softening via reduced expression of multiple cell wall–associated genes (in both locule and pericarp tissues), a number of which have promoter sequences capable of SlLOB1 interaction and which are activated when *SlLOB1* is expressed ectopically in tomato leaves. *SlLOB1* overexpression plants had contrasting phenotypes and gene expression, further supporting the role of this TF in directing fruit textural changes. While *SlLOB1* ectopic expression plants displayed numerous nonfruit phenotypes, the limitation of fruit phenotypes in RNAi repression lines is consistent with *SlLOB1* expression primarily in this tissue, suggesting a primary role in fruit ripening. In addition to cell wall phenotypes, *SlLOB1* repression enhances carotenoid accumulation, possibly via ethylene and/or HSP stabilization of carotenoid pathway enzymes. These results raise the possibility of enhancing both texture and nutritional quality via targeted repression or selection of low-expression *SlLOB1* alleles during breeding.

Materials and Methods

Plant Material. Tomato plants were greenhouse grown under a 16-h light (26 to 29 °C) 8-h dark (17 to 20 °C) cycle. Fruit were tagged at 1-cm diameter (8 to 9 d post-anthesis [DPA]), and IMG (20 DPA), MG (35 DPA), BR (39 DPA), B7 (red ripe), and B15 (overripe) were harvested. Pericarp and locular gel were separated and frozen in liquid nitrogen and stored at −80 °C. Seeds were extracted from RR fruit.

Metabolite and Molecular Analysis. Details of metabolite (ethylene and texture measurements, carotenoid extraction and quantification, cuticle staining, cell wall immunohistochemistry, cell wall material extraction, and wall swelling analysis) and molecular (DNA constructs and tomato transformation, RNA-seq library construction and qRT-PCR, Illumina read processing and GO enrichment analysis, protein gel-blot analysis, and dual luciference assay) analyses are provided in *SI Appendix*. All primers used in this work are listed in *SI Appendix*, Table S3.

Data Availability. All study data are included in the article and/or supporting information.

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1. H. J. Klee, J. J. Giovannoni, Genetics and control of tomato fruit ripening and quality attributes. *Annu. Rev. Genet.* **45**, 41–59 (2011).
2. J. Giovannoni, C. Nguyen, B. Ampofo, S. Zhong, Z. Fei, The epigenome and transcriptional dynamics of fruit ripening. *Annu. Rev. Plant Biol.* **68**, 61–84 (2017).
3. G. Tucker *et al*., Ethylene and fruit softening. *Food Qual. Saf* **1**, 253–267 (2017).
4. D. Wang, T. H. Yeats, S. Uluisik, J. K. C. Rose, G. B. Seymour, Fruit softening: Revisiting the role of pectin. *Trends Plant Sci.* **23**, 302–310 (2018).
5. D. Dellapenna, D. S. Kates, A. B. Bennett, Polygalacturonase gene expression in Rutgers, *rin*, *nor*, and *Nr* tomato fruits. *Plant Physiol.* **85**, 502–507 (1987).

6. M. G. Kramer, K. Redenbaugh, Commercialization of a tomato with an antisense polygalacturonase gene: The FLAVR SAVR tomato story. *Euphytic.* **79**, 293–297 (1994).
7. D. A. Brummell, B. D. Hall, A. B. Bennett, Antisense suppression of tomato endo-1,4-β-glucanase Cel2 mRNA accumulation increases the force required to break fruit abscission zones but does not affect fruit softening. *Plant Mol. Biol.* **40**, 615–622 (1999).
8. M. Saladié, J. K. C. Rose, D. J. Cosgrove, C. Catalá, Characterization of a new xyloglucan endotransglucosylase/hydrolase (XTH) from ripening tomato fruit and implications for the diverse modes of enzymic action. *Plant J.* **47**, 282–295 (2006).
9. L. F. Goulao, C. M. Oliveira, Cell wall modifications during fruit ripening: When a fruit is not the fruit. *Trends Food Sci. Technol.* **19**, 4–25 (2008).
10. D. A. Brummell *et al*., Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. *Plant Cell* **11**, 2203–2216 (1999).
11. S. Uluisik *et al*., Genetic improvement of tomato by targeted control of fruit softening. *Nat. Biotechnol.* **34**, 950–952 (2016).
12. J. Vrebalov *et al*., A MADS-box gene necessary for fruit ripening at the tomato *ripening-inhibitor* (*rin*) locus. *Science* **296**, 343–346 (2002).
13. K. Manning *et al*., A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nat. Genet.* **38**, 948–952 (2006).
14. J. J. Giovannoni, S. Tanksley, J. Vrebalov, E. Noensie, NOR gene for use in manipulation of fruit quality and ethylene response. US Patent No. 6762347 (2004).
15. Y. Ito *et al*., Re-evaluation of the *rin* mutation and the role of *RIN* in the induction of tomato ripening. *Nat. Plants* **3**, 866–874 (2017).
16. S. Li *et al*., The *RIN-MC* fusion of MADS-box transcription factors has transcriptional activity and modulates expression of many ripening genes. *Plant Physiol.* **176**, 891–909 (2018).
17. M. Y. Chung *et al*., A tomato (*Solanum lycopersicum*) *APETALA2/ERF* gene, *SlAP2a*, is a negative regulator of fruit ripening. *Plant J.* **64**, 936–947 (2010).
18. Y. Shinozaki *et al*., High-resolution spatiotemporal transcriptome mapping of tomato fruit development and ripening. *Nat. Commun.* **9**, 364 (2018).
19. E. M. Eriksson *et al*., Effect of the *Colorless non-ripening* mutation on cell wall biochemistry and gene expression during tomato fruit development and ripening. *Plant Physiol.* **136**, 4184–4197 (2004).
20. S. Zhong *et al*., Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nat. Biotechnol.* **31**, 154–159 (2013).
21. P. W. Oeller, M. W. Lu, L. P. Taylor, D. A. Pike, A. Theologis, Reversible inhibition of tomato fruit senescence by antisense RNA. *Science* **254**, 437–439 (1991).
22. S. Picton, S. L. Barton, M. Bouzayen, A. J. Hamilton, D. Grierson, Altered fruit ripening and leaf senescence in tomatoes expressing an antisense ethylene forming enzyme transgene. *Plant J.* **3**, 469–481 (1993).
23. J. Q. Wilkinson, M. B. Lanahan, H. C. Yen, J. J. Giovannoni, H. J. Klee, An ethylene-inducible component of signal transduction encoded by *never-ripe*. *Science* **270**, 1807–1809 (1995).
24. Y. Li *et al*., *LeERF1* positively modulated ethylene triple response on etiolated seedling, plant development and fruit ripening and softening in tomato. *Plant Cell Rep.* **26**, 1999–2008 (2007).
25. N. H. Chapman *et al*., High-resolution mapping of a fruit firmness-related quantitative trait locus in tomato reveals epistatic interactions associated with a complex combinatorial locus. *Plant Physiol.* **159**, 1644–1657 (2012).
26. Tomato Genome Consortium, The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485**, 635–641 (2012).
27. Y. Yang, X. Yu, P. Wu, Comparison and evolution analysis of two rice subspecies *LATERAL ORGAN BOUNDARIES* domain gene family and their evolutionary characterization from *Arabidopsis*. *Mol. Phylogenet. Evol.* **39**, 248–262 (2006).
28. C. Xu, F. Luo, F. Hochholdinger, LOB domain proteins: Beyond lateral organ boundaries. *Trends Plant Sci.* **21**, 159–167 (2016).
29. H. W. Lee, M. J. Kim, N. Y. Kim, S. H. Lee, J. Kim, LBD18 acts as a transcriptional activator that directly binds to the *EXPANSIN14* promoter in promoting lateral root emergence of Arabidopsis. *Plant J.* **73**, 212–224 (2013).
30. H. W. Lee, M. J. Kim, M. Y. Park, K. H. Han, J. Kim, The conserved proline residue in the LOB domain of LBD18 is critical for DNA-binding and biological function. *Mol. Plant* **6**, 1722–1725 (2013).
31. L. Ba *et al*., The banana *MaLBD* (LATERAL ORGAN BOUNDARIES DOMAIN) transcription factors regulate *EXPANSIN* expression and are involved in fruit ripening. *Plant Mol. Biol. Report.* **32**, 1103–1113 (2014).
32. X. F. Wang *et al*., Identification, evolution and expression analysis of the LBD gene family in tomato. *China Agri. Sci.* **46**, 2501–2513 (2013).
33. L. Liu *et al*., CRISPR/Cas9 targeted mutagenesis of *SlLBD40*, a lateral organ boundaries domain transcription factor, enhances drought tolerance in tomato. *Plant Sci.* **301**, 110683 (2020).
34. C. Orfila *et al*., Altered middle lamella homogalacturonan and disrupted deposition of (1–>5)-alpha-L-arabinan in the pericarp of Cnr, a ripening mutant of tomato. *Plant Physiol.* **126**, 210–221 (2001).
35. S. Fragkostefanakis, F. Dandachi, P. Kalaitzis, Expression of arabinogalactan proteins during tomato fruit ripening and in response to mechanical wounding, hypoxia and anoxia. *Plant Physiol. Biochem.* **52**, 112–118 (2012).
36. J. J. Giovannoni, D. DellaPenna, A. B. Bennett, R. L. Fischer, Expression of a chimeric polygalacturonase gene in transgenic *rin* (ripening inhibitor) tomato fruit results in polyuronide degradation but not fruit softening. *Plant Cell* **1**, 53–63 (1989).
37. D. Wang *et al*., Characterization of CRISPR mutants targeting gene modulating pectin degradation in ripening tomato. *Plant Physiol.* **179**, 544–557 (2019).
38. T. Isaacson *et al*., Cutin deficiency in the tomato fruit cuticle consistently affects resistance to microbial infection and biomechanical properties, but not transpirational water loss. *Plant J.* **60**, 363–377 (2009).
39. A. Mabuchi, K. Soga, K. Wakabayashi, T. Hoson, Phenotypic screening of Arabidopsis T-DNA insertion lines for cell wall mechanical properties revealed *ANTHOCYANIN-LESS2*, a cell wall-related gene. *J. Plant Physiol.* **191**, 29–35 (2016).
40. Q. Du *et al*., Activation of *miR165b* represses *AtHB15* expression and induces pith secondary wall development in Arabidopsis. *Plant J.* **83**, 388–400 (2015).
41. C. Zhong *et al*., AtGASA6 serves as an integrator of gibberellin-, abscisic acid- and glucose-signaling during seed germination in *Arabidopsis*. *Plant Physiol.* **169**, 2288–2303 (2015).
42. S. Gille, M. Pauly, O-acetylation of plant cell wall polysaccharides. *Front Plant Sci.* **3**, 12 (2012).
43. Y. Hu *et al*., *Lateral organ boundaries 1* is a disease susceptibility gene for citrus bacterial canker disease. *Proc. Natl. Acad. Sci. U.S.A.* **111**, E521–E529 (2014).
44. Y. S. Yordanov, S. Regan, V. Busov, Members of the LATERAL ORGAN BOUNDARIES DOMAIN transcription factor family are involved in the regulation of secondary growth in Populus. *Plant Cell* **22**, 3662–3677 (2010).
45. J. K. C. Rose, A. B. Bennett, Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: Parallels between cell expansion and fruit ripening. *Trends Plant Sci.* **4**, 176–183 (1999).
46. Y. Gao *et al*., Diversity and redundancy of the ripening regulatory networks revealed by the fruitENCODE and the new CRISPR/Cas9 *CNR* and *NOR* mutants. *Hortic. Res.* **6**, 39 (2019).
47. J. Kropat *et al*., A regulator of nutritional copper signaling in *Chlamydomonas* is an SBP domain protein that recognizes the GTAC core of copper response element. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 18730–18735 (2005).
48. A. Husbands, E. M. Bell, B. Shuai, H. M. Smith, P. S. Springer, LATERAL ORGAN BOUNDARIES defines a new family of DNA-binding transcription factors and can interact with specific bHLH proteins. *Nucleic Acids Res.* **35**, 6663–6671 (2007).
49. J. Zhang *et al*., An AGAMOUS MADS-box protein, SlMBP3, regulates the speed of placenta liquefaction and controls seed formation in tomato. *J. Exp. Bot.* **70**, 909–924 (2019).
50. I. Neta-Sharir, T. Isaacson, S. Lurie, D. Weiss, Dual role for tomato heat shock protein 21: Protecting photosystem II from oxidative stress and promoting color changes during fruit maturation. *Plant Cell* **17**, 1829–1838 (2005).